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# Outbreaks of Gastroenteritis in Elderly Nursing Homes and Retirement Facilities Associated With Human Caliciviruses

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Eleven outbreaks of acute gastroenteritis, eight of which were in nursing homes or retirement facilities, were reported in Virginia during the winter of 1993–1994. Serum samples (four outbreaks) and stool samples (two outbreaks) from involved people were tested for human calicivirus (HuCV) infection by enzyme immune assays (EIAs) using recombinant Norwalk virus (rNV) and Mexico virus (rMX) capsid antigens and reverse transcription-polymerase chain reaction (RT-PCR). Of the 31 pairs of acute and convalescent serum specimens tested, 24 had a fourfold or more titer increase to rMX and 4 responded to rNV. In all four outbreaks, the geometric mean titers (GMTs) against rMX were significantly higher than those against rNV in the covalescent, but not in the acute phase of illness. The antibody response to rMX among these patients was also higher than to rNV (summary mean 32-fold increase vs. 0.7-fold increase, respectively, $P < .001$). Antigen was detected in 5 of 21 stool specimens tested by the rMX EIA, RNA in 12 of 17 stool specimens tested by RT-PCR, and small round structured virus (SRSV) particles in 12 of 21 by electron microscopy (EM); none were positive by the rNV EIA. Sequence analysis of the RT-PCR-amplified products from the viral RNA polymerase region revealed 92–93% amino acid identity with Snow Mountain agent (SMA), 86% with MX, 58–59% with NV, and 31–32% with Sapporo HuCV, suggesting that these viruses belong to the SMA HuCV genogroup.



**KEY WORDS:** human calicivirus, Snow Mountain agent, outbreak of gastroenteritis, enzyme immunoassay, rMX antigen, diarrhea

## INTRODUCTION

Human caliciviruses (HuCVs) include a group of morphologically, genetically, and antigenically diverse positive-stranded RNA viruses associated with acute nonbacterial gastroenteritis in humans. Before the cloning of the prototype Norwalk virus (NV) genome [Jiang et al., 1990, 1993], HuCVs generally were called Norwalk-like or Norwalk-related viruses [Adler and Zickl, 1969; Kapikian and Chanock, 1985] or named for the location of collection, such as Snow Mountain agent [SMA; Dolin et al., 1982], Hawaii agent [HA; Dolin et al., 1975; Thornhill et al., 1977], or Sapporo calicivirus [Nakata et al., 1983]. Two HuCV morphologic types have been described by electron microscopy (EM) examination: small round structured viruses (SRSVs) and morphologically typical caliciviruses [Caul and Appleton, 1982]. Based upon sequences of the RNA-dependent RNA-polymerase region of the genomes, HuCVs now have been classified into four genogroups: NV, SMA, Sapporo, and hepatitis E virus (HEV) genogroups [Jiang et al., 1995a; Matson et al., 1995; Wang et al., 1994]. All four genogroups have a worldwide distribution, although the SMA genogroup appears to be the predominant cause of gastroenteritis at present in most regions studied. Analysis of the sequences of newly detected strains within the SMA genogroup shows that the SMA genogroup can be further divided into subgenogroups [Jiang et al., 1995c]. The Sapporo genogroup also is genetically diverse and can be further divided [Berke et al., 1995]. Therefore, the current genetic classification of HuCVs is preliminary and further characterization of sequences within and among genogroups is necessary.

Antigenic typing of HuCV is also not understood fully due to the inability to passage HuCVs in cell culture

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or animal models and to the lack of sensitive and specific assays for HuCVs. Using immune electron microscopy (IEM), solid phase IEM, or cross-challenge studies in volunteers, four antigenic types in the United Kingdom [SRSV UK1–4; Lewis, 1990, 1992], six antigenic types in the United States [Lewis et al., 1995; Wyatt et al., 1974], and up to nine antigenic types in Japan [Okada et al., 1990] have been described. Because all these classification schemes used human reagents that are of unknown specificity and sensitivity and are in limited supply, a universal antigenic classification scheme of HuCVs is lacking.

The recent development of assays based on the baculovirus-expressed recombinant HuCV antigens showed promise for diagnosis and typing of the family [Graham et al., 1994; Jiang et al., 1992a, 1995a,b,c; Lew et al., 1994]. The recombinant NV (rNV) and MX (rMX) antigen-detection EIAs are group specific and do not detect Sapporo genogroup HuCVs [Jiang et al., 1995a,c]. The rMX antigen enzyme immunoassay (EIA) also appeared to be highly specific in that it detected genetically closely related strains within the SMA genogroup [Jiang et al., 1995c]. The antibody detection EIAs based on the expressed rNV and rMX viral capsids exhibit a low level of cross-reaction between the two genogroups [Jiang et al., 1995b]. The significantly high immune response of volunteers to homologous viruses suggests that the antibody EIAs can be used to differentiate HuCVs causing infection. In this study we applied the rNV and rMX EIAs to four outbreaks of acute gastroenteritis that occurred in nursing homes and retirement centers in Virginia during the winter of 1993–1994. Virologic and serologic evidence suggested that SMA genogroup viruses were involved in these outbreaks.

## MATERIALS AND METHODS

### Outbreak Investigation

Investigations were initiated by either epidemiologists from local health departments or from the state office of epidemiology and included recording of demographic data from all persons with symptoms or signs of illness and use of a questionnaire to gather data from all residents and employees, whether ill or well. Acute and convalescent serum samples and stool specimens were collected from symptomatic individuals. Stool specimens were tested for bacterial enteric pathogens by the state laboratory. Stool and serum specimens were split and sent to the Centers for Disease Control and Prevention (CDC) and to the Center for Pediatric Research for testing for viral agents. Stool and serum specimens were stored at −20°C before testing.

### Detection of Serum Antibody to MX Virus and NV

Acute and convalescent serum pairs were titered in twofold dilution for antibodies to rMX and rNV by EIA [Jiang et al., 1992a, 1995b] on the same plate. Sera collected from a volunteer before and after infection with NV virus [Graham et al., 1994] were included as internal controls on each plate. A seroresponse was defined as a fourfold or greater rise in antibody titer.

### Detection of Viral Antigens

Stool specimens collected during episodes of diarrhea were tested for viral antigens by the rNV and rMX EIAs [Graham et al., 1994; Jiang et al., 1995c]. Each stool suspension (5% w/v in 0.01 M phosphate buffer saline) was tested against rabbit pre- and post-hyperimmune antiserum to the respective recombinant viral antigens. A positive (post)/negative (pre) ratio of $\geq 2$ and an OD value $>0.1$ in the well coated with rabbit post-serum was considered a positive result.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Detection of Viral Genome

Primer pairs NV36/35 and NV36/39, each based upon the sequence of the RNA-dependent RNA-polymerase region of the NV genome, were used in an RT-PCR to detect HuCV genome in stool specimens. NV36/35 produces a product of 470 base cDNA and NV36/39 produces a 400 base cDNA.

The cetyltrimethylammonium bromide (CTAB) method was used to extract RNA from stool specimens for RT-PCR [Jiang et al., 1992b]. RT was performed with a slight modification from the previously described technique. In a reaction volume of 50 μL, 5 μL of extracted RNA, 5 units of avian myeloblastosis virus reverse transcriptase, 1.0 μM primer, and 5 units of RNAsin were mixed in 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 3.3 mM each of dATP, dCTP, dGTP, and dTTP). The RT reaction was performed for 1 hr at 42°C. After incubation, 50 μL of 1 × PCR reaction mixture containing the second primer and 5 units of Taq polymerase were added. The 40 PCR cycles included 1 min at 94°C, 1 min at 45°C, and 90 sec at 72°C. The RT-PCR products were analyzed by agarose gel electrophoresis and visualized by UV light illumination.

### Sequence Analysis of the RT-PCR-Amplified Products

The RT-PCR-amplified products from the stool specimens were cloned using the p-GEMT cloning kit (Promega, Madison, WI) and sequenced. Sequences were analyzed using PC/GENE software, version 6.90 (Intelligentities, Mountain View, CA).

### Statistical Analysis

Statistical analysis of data was performed using Epi Info Version 6.0 (Centers for Disease Control and Prevention, Atlanta, GA; World Health Organization, Geneva, Switzerland).

## RESULTS

### Results of the Epidemiologic Investigation

Eleven outbreaks of acute gastroenteritis occurred in Virginia during the winter of 1993–1994. Four of the 11 outbreaks were investigated, three of which occurred within a 30-mile radius of Richmond and the fourth

outbreak occurred in a nursing home in western Virginia. The epidemiologic investigations revealed attack rates ranging from 64 to 92%. The most common symptoms included vomiting (75–92%), nausea (96–100%), and diarrhea (48–100%, often watery). Fatigue and fever also were noted commonly. Mean duration of illness ranged from 24 to 29 hr. In the outbreak involving a health care clinic, a potato-egg salad included in a box lunch was the suspected vehicle of transmission. In the other three nursing/retirement home investigations, no common food source could be identified.

### Seroresponses as detected by rMX and rNV EIAs

A total of 31 serum pairs were collected in the four outbreaks and tested for anti-HuCV antibodies (Table I). Seven patients did not respond to either rMX or rNV, 4 responded to both rMX and rNV, 20 responded to rMX only, and none responded to rNV only (Table I). Cases in all four outbreaks exhibited the same pattern of preferential seroresponse to rMX. The geometric mean titers (GMTs) against rMX antigen were significantly higher in the sera collected during convalescent than in the sera collected during the acute phase of illness in all four outbreaks (Table II), indicating these outbreaks were associated with the SMA genogroup. There were no significant differences in geometric mean antibody titers against rNV between sera collected in acute and convalescent phases of illness from patients in all four outbreaks (Table II). In each separate outbreak and in all four outbreaks combined, the mean rise in antibody titer to rMX was significantly higher than to rNV antigen (summary mean 32-fold vs. 0.7-fold increase, $P < .001$).

While antibody assays using different antigens may not be comparable, when acute serum titers were compared, the GMTs against rMX and rNV in three of four outbreaks were similar ($P = .49, .05, .21, .38$, respectively), suggesting similar previous exposure to both NV and SMA. When the convalescent serum titers were compared, the GMTs against rMX were significant higher than against rNV in three of four outbreaks ($P = .005, .003, .19, .006$, respectively), indicating that the increase in titers was rMX specific.

### Detection of Viruses in Stool Specimens by Antigen EIAs, RT-PCR, and EM

Twenty-one stool specimens from two outbreaks were tested and five (outbreak 1: 3 of 11 and outbreak 2: 2 of 10) were positive by the rMX antigen EIA (Table III). None were positive by the rNV antigen EIA. Seventeen of the 21 stool specimens were tested by RT-PCR. Twelve (outbreak 1: 6/9 and outbreak 2: 6/8) were positive by primer pair 36/39 and nine (outbreak 1: 6 of 9 and outbreak 2: 3/8) were positive by primer pair 36/35 (Table III). When these stool specimens were examined using EM, SRSV-like particles were found in 12 of 21 stool specimens (Table III).

### Sequence Analysis of the RT-PCR Products

Two RT-PCR products from each of the two outbreaks were cloned and sequenced. Sequence analysis showed that all four viruses had sequence characteristics typical of HuCVs, including a continuous open reading frame in the plus-sense strand and RNA-dependent RNA-polymerase amino acid motifs in the expected distances from the primers (data not shown). The sequences of the two viruses in each outbreak were identical and there was a 99% nucleotide sequence identity between the outbreaks. The two outbreak strains shared 92 or 93% amino acid sequence identity with SMA, 86% with MX virus, 58 or 59% with NV, and 31 or 32% with Sapporo virus (Table IV). Comparison of these four sequences with our recently identified SMA-like HuCVs from child care centers in Norfolk, VA, showed that all four viruses belong to the SMA-like subgroup rather than the MX-like subgroup within the SMA genogroup [18].

## DISCUSSION

This study describes the utility of newly developed recombinant EIA and RT-PCR methods for investigation of outbreaks of HuCV-associated acute gastroenteritis. The rNV EIAs have been demonstrated to be useful for diagnosis of NV infection in volunteer studies and in large surveys of HuCVs from different countries [Gray et al., 1993; Jiang et al., 1995a; Lew et al., 1994; Numata et al., 1994; Parker et al., 1993, 1994; Treanor et al., 1993]. The rMX EIAs were developed recently [Jiang et al., 1995b,c] and their application to field studies remains to be evaluated. This study is the first to use both assays to investigate outbreaks associated with HuCVs. The successful characterization of the causative strain by both virologic and serologic parameters suggests that the new EIAs are useful techniques for large-scale studies of the epidemiology of HuCV infection.

In our previous survey of HuCVs in different countries by rNV EIA and RT-PCR, strains in the NV genogroup were found rarely but SMA genogroup HuCVs were detected frequently [Jiang et al., 1995a]. This observation has now been repeated by rMX EIAs that are specific for SMA-like HuCVs. For example, in Norfolk, from November 1993 through November 1994, we tested over 1,200 diarrhea stool specimens from children attending day care centers monitored weekly for diarrhea. Twenty-three diarrhea stool specimens from 21 children attending five day care centers were found to contain SMA-like HuCVs by both EIA and RT-PCR [Jiang et al., 1995c]. Using the same assays, SMA-like viruses also were found in the United Kingdom [Cubitt and Jiang, 1996] and South Africa [Wolfaardt et al., 1996]. In the United Kingdom, both SMA-like and MX-like subgroup viruses have been found periodically since 1982 [Cubitt and Jiang, 1996]. Therefore, we hypothesize that HuCVs may follow a pattern in that predominant strain(s) of HuCVs may change over time similar to what occurs with many other viral pathogens. The

TABLE I. Titers of Serum Antibodies (Ab) to Baculovirus-Expressed rMX and rNV Capsid Antigens Among Patients in Outbreaks of Gastroenteritis

| Outbreaks | Patients | Ab to rMX | | Fold increase | Ab to rNV | | Fold increase | *P* value* |
|---|---|---|---|---|---|---|---|---|
| | | Acute | Convalescent | | Acute | Convalescent | | |
| 1 | 1 | 800 | 12,800 | 16 | 1,600 | 3,200 | 2 | |
| | 2 | 6,400 | 102,400 | 16 | 1,600 | 6,400 | 4 | |
| | 3 | 1,600 | 25,600 | 16 | 1,600 | 1,600 | 0 | |
| | 4 | 800 | 1,600 | 2 | 400 | 400 | 0 | |
| | 5 | 1,600 | 6,400 | 4 | 3,200 | 6,400 | 2 | |
| | 6 | 1,600 | 25,600 | 16 | 400 | 1,600 | 4 | |
| | 7 | 1,600 | 25,600 | 16 | 3,200 | 3,200 | 0 | |
| | 8 | 1,600 | 102,400 | 64 | 1,600 | 1,600 | 0 | |
| | Number with response | | | 7/8 | | | 2/8 | .04 |
| 2 | 9 | 800 | 1,600 | 2 | 400 | 400 | 0 | |
| | 10 | 6,400 | 25,600 | 4 | 1,600 | 1,600 | 0 | |
| | 11 | 1,600 | 25,600 | 16 | 1,600 | 1,600 | 0 | |
| | 12 | 6,400 | 25,600 | 4 | 400 | 400 | 0 | |
| | 13 | 6,400 | 6,400 | 0 | 400 | 400 | 0 | |
| | 14 | 6,400 | 25,600 | 4 | 1,600 | 1,600 | 0 | |
| | 15 | 800 | 3,200 | 4 | 1,600 | 1,600 | 0 | |
| | Number with response | | | 5/7 | | | 0/7 | .02 |
| 3 | 16 | 400 | 25,600 | 64 | 1,600 | 1,600 | 0 | |
| | 17 | 400 | 6,400 | 16 | 100 | 100 | 0 | |
| | 18 | 200 | 800 | 4 | 400 | 400 | 0 | |
| | 19 | 400 | 1,600 | 4 | 400 | 400 | 0 | |
| | 20 | 400 | 400 | 0 | 1,600 | 1,600 | 0 | |
| | 21 | 400 | 800 | 2 | 800 | 800 | 0 | |
| | Number with response | | | 4/6 | | | 0/6 | .06 |
| 4 | 22 | 800 | 6,400 | 8 | 100 | 100 | 0 | |
| | 23 | 100 | 100 | 0 | 400 | 400 | 0 | |
| | 24 | 1,600 | 102,400 | 64 | 100 | 100 | 0 | |
| | 25 | 400 | 6,400 | 16 | 400 | 400 | 0 | |
| | 26 | 400 | 6,400 | 16 | 400 | 400 | 0 | |
| | 27 | 400 | 102,400 | 256 | 1,600 | 6,400 | 4 | |
| | 28 | 1,600 | 102,400 | 64 | 400 | 800 | 2 | |
| | 29 | 400 | 6,400 | 16 | 800 | 800 | 0 | |
| | 30 | 400 | 102,400 | 256 | 1,600 | 6,400 | 4 | |
| | 31 | 1,600 | 1,600 | 0 | 100 | 100 | 0 | |
| | Number with response | | | 8/10 | | | 2/10 | .02 |

*By chi square with Yate's correction or Fisher's exact test. $P < .001$ for summary Mantel-Haenzol chi square for all four outbreaks.

TABLE II. GMTs of Serum Antibodies to rMX and rNV Antigens in Patients Involved in Four Outbreaks of Acute Gastroenteritis in Virginia

| Outbreaks | GMTs against | | | | | |
|---|---|---|---|---|---|---|
| | rMX | | | rNV | | |
| | Acute | Convalescent | *P* value | Acute | Convalescent | *P* value |
| 1 | 1,552 | 18,820 | .002 | 1,097 | 1,911 | .3 |
| 2 | 2,896 | 10,809 | .063 | 832 | 832 | 1.0 |
| 3 | 362 | 2,048 | .008 | 549 | 549 | 1.0 |
| 4 | 549 | 10,809 | .003 | 362 | 832 | .7 |
| Total | 955 | 9,410 | .001 | 676 | 832 | .5 |

NV genogroup was common in the 1970s, but now is rare; the SMA genogroup has been predominant since the 1980s. This hypothesis is based upon limited studies utilizing the newly developed EIAs and RT-PCR; continued surveys are necessary to confirm this hypothesis.

The age groups of people who may be affected by the SMA genogroup HuCVs remain to be determined. Earlier studies indicated SMA-like viruses mainly caused outbreaks of gastroenteritis in school-aged children and adults [Kapikian and Chanock, 1990]. The studies in child day care centers showed that SMA-like viruses commonly infect young children before they attend school [Jiang et al., 1995c]. The studies described in this report suggested that the SMA genogroup can infect the elderly. Therefore, SMA-like viruses are common in both young children and adults. These two studies also showed that the SMA genogroup can cause both sporadic episodes and outbreaks of gastroenteritis. Recently, we have found the SMA genogroup associated with acute gastroenteritis in 2% of children hospitalized

TABLE III. Detection of HuCVs in Stool Specimens From Patients Involved in Two Outbreaks of Gastroenteritis in Nursing Homes in Virginia by RT-PCR, EIA, and EM*

| Outbreaks | Sample no. | RT-PCR p36/39 | RT-PCR p36/35 | rMX EIA | SRSV + by EM |
|---|---|---|---|---|---|
| 1 | 1 | NT[a] | NT | 0.1 | – |
| | 2 | NT | NT | **2.7** | – |
| | 3 | + | + | 1.8 | + |
| | 4 | + | + | 1.1 | + |
| | 5 | – | + | 1.0 | – |
| | 6 | + | + | 1.6 | + |
| | 7 | – | – | 1.3 | + |
| | 8 | + | + | **2.7** | + |
| | 9 | – | – | 1.1 | – |
| | 10 | + | + | **2.8** | + |
| | 11 | + | – | 1.5 | + |
| Total pos/tested | | 6/9 | 6/9 | 3/11 | 7/11 |
| 2 | 12 | + | – | 1.3 | – |
| | 13 | – | – | 1.0 | – |
| | 14 | + | + | **2.2** | + |
| | 15 | + | + | 1.6 | + |
| | 16 | NT | NT | 1.6 | + |
| | 17 | – | – | 2.3[b] | – |
| | 18 | + | – | 1.9 | + |
| | 19 | + | + | 2.5[b] | + |
| | 20 | + | – | **2.5** | – |
| | 21 | NT | NT | 2.5[b] | – |
| Total pos/tested | | 6/8 | 3/8 | 2/10 | 5/10 |

*All stool specimens were tested by rNV EIA and none were positive.
[a]NT, not tested.
[b]These specimens were considered to be negative due to the OD value < 0.1 of these specimens in the wells coated with the rabbit postimmunization serum.
Bold numbers indicate positive specimens which had an OD value > 0.1 of the specimens in the wells coated with the rabbit postimmunization serum and a P/N ratio ≥ 2.0.

TABLE IV. Genetic and Antigenic Relationships of HuCVs Determined by Type-Specific EIAs and RT-PCR

| Virus | Amino acid identity (%) to NV | SMA | Sapporo | MX | EIA (+/tested) rNV | rMX |
|---|---|---|---|---|---|---|
| 8FIIa NV/68/US[a] | 100 | 57 | 30 | 58 | + | – |
| SMA/76/US[a] | 57 | 100 | 32 | 89 | – | +(2/8) |
| HA/71/US[a] | 61 | 91 | 33 | 83 | – | +(1/8) |
| VA-1/94/US[b] | 59 | 93 | 32 | 86 | – | +(3/11) |
| VA-2/94/US[b] | 58 | 92 | 31 | 86 | – | +(2/10) |
| Nfk 6610/94/US[c] | 60 | 93 | 32 | 88 | – | + |
| MX 34/89/Mex[a] | 58 | 89 | 30 | 100 | – | + |
| Nfk 1135/94/US[c] | 60 | 90 | 32 | 97 | – | + |
| Nfk 1184/94/US[c] | 60 | 90 | 32 | 97 | – | + |
| Nfk 770R/94/US[c] | 60 | 90 | 32 | 97 | – | + |
| Nfk 5230/94/US[c] | 59 | 89 | 30 | 94 | – | + |
| Nfk 5432/94/US[c] | 58 | 88 | 30 | 93 | – | + |
| HuCV/Sapp/82/J[a] | 30 | 31 | 100 | 31 | – | – |

[a]Prototype Norwalk, Snow Mountain, Hawaii, Mexico, and Sapporo HuCVs.
[b]Virus strains detected in the Virginia nursing home.
[c]Virus strains detected in Norfolk child care centers.

for diarrhea (unpublished observations), indicating that the SMA genogroup is a cause of severe gastroenteritis in children. In conclusion, our understanding of SMA genogroup epidemiology is still changing and further studies with the new assays are necessary.

The outbreak studies described in this paper also provided support for the classification of the SMA genogroup into genetic and antigenic subgroups. The high level of seroresponse to rMX in all four outbreaks suggested a SMA genogroup strain as a cause of the outbreaks. The sequence of the viruses detected from these outbreaks showed clearly that they belong to the subgroup containing the prototype SMA. In contrast to the serologic results, viral antigen was detected at a low rate (5 of 21) in stool specimens from patients in the outbreaks by the rMX antigen EIA, a rate significantly

lower than that of RT-PCR or EM. This result is similar to previous studies of testing stool specimens from volunteers infected with SMA and HA, in which low rates of antigen detection were obtained [Jiang et al., 1995c]. The prototypes SMA and HA tentatively have been classified into the same SMA subgroup, while the MX virus is in another subgroup, a distinction confirmed by phylogenetic tree construction based upon primary sequence [Berke et al., 1995]. Therefore, the low rate of detection by rMX EIA of viruses in the SMA/HA subgroup suggested that the two subgroups may also have some distinct antigens.

The genetic and antigenic diversities of HuCVs have also been described by others. Based upon the analysis of the RNA polymerase sequence, Ando et al. [1995] suggested that both NV and SMA genogroups can be divided further. By using the same rMX EIA, Hale et al. [1996] described antigenically high, middle, and low-reactor SMA-like viruses in stool specimens from England and the variation was correlated with their degree of genetic identity. These findings highlight the need for clarifying the specificity of the new immunologic assays for the detection of HuCVs. Currently, there are EIAs based upon two baculovirus-expressed viral capsid antigens that are available for diagnosis of HuCVs. Development of similar assays to other antigenic types and development of monoclonal antibodies against these antigens for more specific assays are necessary. These developments are now in progress and include the expression of Toronto virus, Hawaii virus, and Desert Storm virus in different laboratories [Monroe and Green, personal communication] and the production of monoclonal antibodies against rNV [Hardy et al., 1996] and rMX antigens [our unpublished data]. A better understanding of the classification and relationships between antigenic and genetic typing of HuCV will occur with these new assays.

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